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Human Cytomegalovirus Glycoprotein B Genotypes in Renal Transplant Recipients

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Based on sequence variation of the glycoprotein B (gB) gene, human cytomegalovirus (HCMV) strains can be classified into four gB genotypes. In a previous study of bone marrow transplant recipients, infection with the gB type 1 correlated with a more favorable clinical outcome than infection with the gB types 2, 3, or 4. The gB type was determined in 60 renal transplant and in 47 bone marrow transplant recipients using PCR and restriction analysis. All HCMV variants in patient specimens could be assigned to one of the four previously described gB types. Two or more specimens obtained from 39 patients were analysed; in 31 of these patients the gB type was the same in all samples. The gB type did not correlate with the clinical outcome or the level of viremia in renal transplant recipients. © 1996 Wiley-Liss, Inc.

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INTRODUCTION

Human cytomegalovirus (HCMV) causes significant morbidity and mortality in immunocompromised patients, although infection of immunocompetent individuals is generally asymptomatic [Suwansirikul et al., 1977]. However, not all immunocompromised patients with HCMV infection develop HCMV-associated disease, which may reflect differences in the degree of immunodeficiency. On the other hand, differences in the biologic behavior of HCMV strains have been described [Brown et al., 1995]. It is possible that genetic variation of functionally important genes may influence the virulence of HCMV strains.

One important component of the HCMV virion is the glycoprotein B (gB) protein. The gB protein is not only the major target for neutralizing antibodies [Cranage et al., 1986], but is also important for virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells [Tugizov et al., 1994, 1995; Navarro et al., 1993]. Certain regions of the gB gene are highly variable between different virus strains. The highly variable region around the protease cleavage site (between amino acid 460 and 461) is involved in the fusion processes [Navarro et al., 1993]. Based on the nucleotide sequence coding for this variable region, a genotyping scheme was proposed [Chou, 1992]. Strains adopt one of four gB genotypes. In one study, the gB genotype was found to correlate with the clinical outcome of HCMV infection in bone marrow transplant recipients. Patients who survived HCMV infection were infected more frequently with gB type 1 virus than those who died [Fries et al., 1994]. This study has not been confirmed so far. However, in one meeting report, the gB genotype 1 was found less frequently in patients with acquired immunodeficiency syndrome (AIDS) and HCMV retinitis than in AIDS patients with asymptomatic HCMV infection [Rasmussen et al., 1995]. A similar correlation was also found in HIV-infected patients studied in Freiburg (Meyer-König et al., manuscript submitted). Although neither study on AIDS patients is published, they provide further evidence that the gB type may influence the virulence of HCMV strains. Additional studies on different patient groups are necessary to determine whether the gB genotype can indeed influence the clinical outcome of HCMV infection.

The frequency distribution of gB genotypes was examined in renal transplant recipients. In contrast to the findings in AIDS patients and bone marrow transplant recipients, no correlation was found between the gB genotype and the clinical outcome of HCMV infection.

MATERIALS AND METHODS

Patients

Between September 1992 and October 1995, 173 patients receiving cadaveric kidney grafts and 97 bone marrow transplant patients were monitored for HCMV infection. Blood and urine samples were obtained weekly from patients hospitalized after transplantation and at more irregular intervals from outpatients during follow-up. Seventy-three renal and 50 bone marrow transplant patients were HCMV DNA-positive in at least one blood

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sample by polymerase chain reaction (PCR); of these 60 and 47 patients, respectively, were included in this study. Patients were observed for at least 6 months after transplantation. To define HCMV infection and HCMV disease, renal transplant patients were assigned to two groups, according to Cheeseman et al. [1979] with minor modifications. Asymptomatic HCMV infection was diagnosed if at least one of the following markers was positive: viral shedding in urine, pp65 antigen, or viral DNA in peripheral blood leukocytes (PBL). Symptomatic HCMV infection was defined as unexplained fever >37.5°C for at least 3 days in combination with at least one positive virological parameter stated above, and at least one of the following features: arthralgia, leukopenia ($<3 \times 10^9$/L), thrombocytopenia ($<150 \times 10^9$/L), liver enzyme elevation (ALT > 50 U/L), pneumonitis without other causes, or gastrointestinal ulceration.

Detection of pp65 Antigen by Indirect Immunofluorescence

The pp65 antigenemia assay was carried out as described by van der Bij et al. [1988]. Briefly, PBL were isolated within 3 hours after removal. Two ml of PBS and 1 ml of a 5% dextran solution (MW 243 kDa) were added to 6 ml of EDTA blood. After sedimentation for 20 minutes at 37°C, the supernatant was harvested and contaminating erythrocytes were lysed in ice cold lysing solution (0.8% NH_4CL pH 7.4) for 10 minutes. Cells were washed twice in PBS, counted, and used for cytospin preparation or PCR.

PBL were adjusted to a concentration of 2×10^6 cells/ml. Cytospin preparations were obtained by centrifugation of 2×10^5 cells onto glass slides using a Cytospin-2 centrifuge (Shandon, Runcorn, UK). Slides were fixed in PBS/5% paraformaldehyde/2% sucrose at room temperature for 10 minutes and washed in PBS/1% FCS and cells were permeabilised with PBS/0.5% NP40/10% sucrose/1% FCS at room temperature for 5 minutes. Slides were rinsed again, air-dried, and stained directly or stored at −80°C.

Slides were stained at 37°C for 30 minutes with a monoclonal antibody to the HCMV pp65 antigen (clone 1C3, Biosoft, Paris, France), washed in PBS, and incubated at 37°C for 30 minutes with goat anti-mouse FITC (Biosoft, Paris, France) diluted 1/100 in PBS/0.01% Evans blue. Slides were washed, mounted in elvanol, and examined. Results were defined as positive if more than 10 cells per slide (2×10^5 leukocytes) were pp65-positive.

Rapid Centrifugation Culture

Rapid centrifugation culture was carried out according to Gleaves et al. [1985]. Briefly, human diploid fibroblasts cultured in a 24-well plate were inoculated with 0.5 ml urine. After centrifugation for 50 minutes at 900*g*, the urine was replaced by fresh medium. Cultures were incubated for 16 hours at 37°C and 5% CO_2, fixed with acetone/methanol (1:1), and stained with a monoclonal antibody to the HCMV p72 antigen (clone E-13, Biosoft, Paris, France) followed by FITC-labeled goat anti-IgG (Biosoft, Paris, France).

DNA Preparation

PBL (2×10^6) were resuspended in 250 μl lysis buffer (10 mM Tris at pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.5% Tween 20, 0.5% NP-40) supplemented with proteinase K (0.2 mg/ml). After incubation (60°C, 120 minutes), the proteinase K was inactivated (95°C, 10 minutes). The crude DNA extract was used for PCR amplification. The DNA of 200 μl urine was extracted using the Qiamp Blood Kit (Quiagen, Chatsworth, USA) according to the manufacturer's recommendations.

gB PCR and Restriction Analysis

A region of high sequence variability in the gB gene was amplified as previously described [Fries et al., 1994]. A first round of amplification was added to improve sensitivity. To overcome possible sequence variations between strains, a mixture of three 5′ primers was used (5′gBout: 5′-GTTCCGAAGCCGAAGACTCG, 5′gBout2: 5′-GCAGCACCTGGCTCTATCG; 5′gBout3: 5′ GCCAGCTCACCTTCTGGG, 3′gB out: 5′-GCACCTTGACGCTGGTTTGG). The first round amplification was performed using 25 μl crude DNA extract and 25 μl PCR mix A [10 mM Tris-Cl at pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, four dNTPs (400 μM each), 1.25 units of Taq polymerase (Promega, Madison, WI, USA)] with 5 pmol of each 5′ primer and 15 pmol of the 3′ primer. For second round amplification, 1 μl of first round amplification product was added to 49 μl of PCR mix B [10 mM Tris at pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, four dNTPs (200 μM each), 1.25 units of Taq polymerase (Promega)] with 5 pmol of each primer. The samples were covered with mineral oil (Sigma, Deisenhofen, Germany), denatured (95°C, 360 seconds), and amplified within 35 cycles (95°C, 90 seconds; 55°C, 120 seconds; 72°C, 60 seconds). Amplification products were analysed by electrophoresis in a 2% agarose gel stained with 0.5 μg/ml ethidium bromide and subjected to restriction analysis using *Hinf*I and *Rsa*I (New England Biolabs, Beverly, MA, USA) as previously described [Fries et al., 1994]. Digested DNA was analysed on 10% polyacrylamide gels. Four distinct gB genotypes were identified by their typical restriction patterns (fragment lengths: 36 to 239 bp).

Statistics

Significance was checked using the χ^2-test.

RESULTS

gB Genotypes in Renal and Bone Marrow Transplant Recipients

The HCMV gB type was determined by PCR amplification of a variable region of the gB gene followed by restriction enzyme analysis. A total of 149 blood and 37 urine samples from 60 renal transplant and 47 bone marrow transplant recipients were analysed. The results are shown in Table I. The frequencies of the gB types 1, 2, and 3 were all very similar in renal transplant recipients. In bone marrow transplant recipients, gB

TABLE I. Frequency of gB Genotypes for Different Patient Groups

gB Genotype	Patient group studied			
	Kidney/Germany	BM/Germany	Organ/USA[a]	BM/USA[b]
1	14 (23%)	20 (43%)	15 (38%)	60 (54%)
2	18 (30%)	16 (34%)	11 (28%)	11 (10%)
3	18 (30%)	6 (13%)	11 (28%)	24 (21%)
4	4 (7%)	0 (0%)	3 (8%)	10 (9%)
Mix	6 (10%)	5 (11%)	0 (0%)	7 (6%)
Totals	60	47	40	112

BM, bone marrow.
[a]Chou and Dennison, 1991; organ transplant recipients.
[b]Fries et al., 1994; BM transplant recipients.

type 3 was found less frequently than the types 1 and 2 ($P = 0.03$).

Only a few patients were infected with gB type 4 or with mixtures of several gB types. Mixtures of gB types were defined as the presence of more than one gB type in a single sample or in different samples from the same patient. For 39 patients more than one specimen was analysed and in eight of these patients the gB types in different samples were not identical. The diagnosis of a mixture of gB types in a single sample was made if the lengths of the restriction fragments corresponded to more than one gB type. In five samples from three patients, which had such atypical restriction patterns, PCRs were carried out on dilutions of the specimens. At higher dilutions the gB types contributing to the mixture could clearly be resolved (data not shown) and we excluded that the non-classifiable restriction patterns were due to atypical gB types. Thus, all HCMV variants in the patients studied could be assigned to one of the four gB types described previously.

gB Genotype and Clinical Outcome

A previous study has suggested that the gB genotypes differ in virulence in bone marrow transplant recipients. The relationship between gB type and severity of disease was difficult to assess in bone marrow transplant recipients because these patients are screened weekly for HCMV viremia by PCR and receive preemptive therapy with ganciclovir as soon as two successive tests are positive. All bone marrow transplant patients in this study thus received ganciclovir. In contrast, renal transplant patients only received antiviral therapy when HCMV infection was symptomatic. For renal transplant recipients it was, therefore, possible to compare gB genotype with clinical outcome of HCMV infection. The patients included in this study were followed-up for at least 6 months after transplantation. Patients were screened weekly for viremia by PCR and antigenemia assay. Altogether 23 patients developed symptomatic HCMV infection and four of these had HCMV-associated organ disease: two patients with pneumonia (one infected with gB type 1, the other with gB type 3) and two patients with gastrointestinal ulceration (one infected with gB type 2, the other with gB type 3). The frequency distribution for the patients with symptomatic and asymptomatic infection are shown in Table II. All symptomatic patients were treated with ganciclovir for 2 weeks. There was no correlation between the gB genotype and the development of symptomatic HCMV infection. Moreover, the level of viremia, as determined by the antigenemia assay, did not differ significantly between gB genotypes (Table III). Thus, the gB genotypes of HCMV did not differ in their pathogenicity in renal transplant recipients.

TABLE II. Renal Transplant Recipients: Glycoprotein B Genotypes in Symptomatic and Asymptomatic HCMV Infection

gB Genotype	Clinical staging[a]	
	Asymptomatic	Symptomatic
1	7	7
2	13	7
3	10	8
4	4	0
Mix	3	1

[a]The definition of symptomatic and asymptomatic HCMV (human cytomegalovirus) infection is given in Materials and Methods.

TABLE III. Glycoprotein B (gB) Genotypes and Level of Viral pp65-Antigenemia in Renal Transplant Recipients

gB genotype	Antigenemia[a]	
	Positive	Negative
1	10	4
2	15	5
3	8	10
4	4	0
Mix	2	2

[a]Antigenemia assay 'positive': more than 10 of 2×10^5 leukocytes were pp65-positive.

DISCUSSION

This study confirms that HCMV variants can be classified into four gB genotypes. In contrast to previous studies on virus isolates, we typed HCMV directly in specimens from patients. This gives a more authentic picture of the gB types present in vivo by omitting possible selection of certain virus types during in vitro cultivation. All variants in the patient specimens could be assigned to one of the four previously described gB types. In contrast to previous studies on bone marrow transplant

recipients and AIDS patients [Rasmussen et al., 1995; Fries et al., 1994], no correlation was found between the gB type and the clinical outcome of HCMV infection in renal transplant recipients.

The frequency distribution of gB types 1, 2, and 3 in renal transplant recipients did not differ significantly from values published previously for organ transplant recipients [Chou and Dennison, 1991] (Table I). The gB type 4 virus and mixtures of several gB types were rarely found in renal and bone marrow transplant recipients (*<10%*). These results are also in agreement with previous studies [Fries et al., 1994; Chou and Dennison, 1991].

In bone marrow transplant recipients, the gB type 3 was found less frequently than the gB types 1 and 2. This difference was not seen in a previous study [Fries et al., 1994], which found that gB type 2 virus was less frequent than gB type 3. This differs significantly from the frequencies for gB types 2 and 3 found in the present study (Table III, $P = 0.02$). However, the study by Fries et al. [1994] is not entirely comparable to ours. First, the HCMV isolates studied were mostly from patients in the United States, while our patients were from southern Germany. It is not known whether there are regional differences in the frequency distribution of gB types. Second, Fries et al. [1994] studied virus isolates rather than typing virus in the patient specimens directly, as we did. Possibly, certain virus types were selected during the process of virus isolation. Third, the strains studied previously were isolated from several sources including biopsies and throat swabs, while we generally used blood and urine specimens for gB typing. Possibly, gB types differ in their cell or tissue tropism. Further studies are necessary to determine which of these three factors can actually influence the frequency distribution of gB types in patients.

In one previous unpublished study, no correlation was found between the gB type and the clinical outcome in liver transplant recipients in Berlin (personal communication, C. Schmidt). This is in agreement with our data in renal transplant recipients. However, it remains unclear why the gB type was found to correlate with the clinical outcome in AIDS patients and bone marrow transplant recipients but not in organ transplant recipients [Fries et al., 1994]. The main factor that influences clinical outcome is the immune status of the patient and clearly all three groups of patients are immunologically distinct. The immune system of organ transplant recipients can suppress HCMV more efficiently than that of bone marrow transplant recipients and AIDS patients and the difference in virulence of gB types may be less relevant in a more immunocompetent host. On the other hand, HCMV is a large and complex virus with a genome that contains over 200 open reading frames [Pereira et al., 1993]. Clearly, the gB protein is not the only factor that could influence the virulence of HCMV. The relative impact of the gB type on the severity of HCMV disease may vary in different patient groups.

In conclusion, in contrast to a previous study in bone marrow transplant recipients and unpublished observations for AIDS patients, we did not find that the gB types of HCMV differed in their pathogenicity in renal transplant recipients. Therefore, the extent to which the gB type can influence clinical outcome of HCMV infection may depend on the underlying type of immunodeficiency.

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